AVIAN DISEASES 52:14–22, 2008

Immunopathology and Cytokine Responses in Broiler Chickens Coinfected with *Eimeria maxima* and *Clostridium perfringens* with the Use of an Animal Model of Necrotic Enteritis

Soon S. Park,[A] Hyun S. Lillehoj,[AB] Patricia C. Allen,[A] Dong Woon Park,[A] Steve FitzCoy,[C] Daniel A. Bautista,[D] and Erik P. Lillehoj[E]

[A]Animal Parasitic Diseases Laboratory, ANRI, BARC, USDA-ARS, Beltsville, MD 20705
[C]Schering-Plough Animal Health, Millsboro, DE 19966
[D]Lasher Poultry Diagnostic Laboratory, University of Delaware, Georgetown, DE 19947
[E]Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD 21201

Received 20 April 2007; Accepted and published ahead of print 7 August 2007

SUMMARY. The incidence of necrotic enteritis (NE) due to *Clostridium perfringens* (CP) infection in commercial poultry has been increasing at an alarming rate. Although pre-exposure of chickens to coccidia infections is believed to be one of the major risk factors leading to NE, the underlying mechanisms of CP virulence remain undefined. The objectives of this study were to utilize an experimental model of NE produced by *Eimeria maxima* (EM) and CP coinfection to investigate the pathologic and immunologic parameters of the disease. Broilers coinfected with EM plus CP exhibited more severe gut pathology compared with animals given EM or CP alone. Additionally, EM/CP coinfection increased the numbers of intestinal CP bacteria compared with chickens exposed to an identical challenge of CP alone. Coinfection with EM and CP repressed nitric oxide synthase gene expression that was induced by EM alone, leading to lower plasma NO levels. Intestinal expression of a panel of cytokine and chemokine genes following EM/CP coinfection showed a mixed response depending on the transcript analyzed and the time following infection. In general, IFN-α, IFN-γ, IL-1β, IL-2, IL-12, IL-13, IL-17, and TGF-β4 were repressed, whereas IL-8, IL-10, IL-15, and LITAF were increased during coinfection compared with challenge by EM or CP alone. These results are discussed in the context of EM and CP to act synergistically to create a more severe disease phenotype leading to an altered cytokine/chemokine response than that produced by infection with the individual pathogens.

RESUMEN. Inmunopatología y respuesta de citoquinas en pollos de engorde coinfectados con *Eimeria maxima* y *Clostridium perfringens* utilizando un modelo animal para enteritis necrótica.

La incidencia de enteritis necrótica debido a la infección por *Clostridium perfringens* se ha incrementado en una tasa alarmante en aves domésticas comerciales. Aun cuando la exposición previa de las aves a infecciones con coccidia se considera uno de los mayores factores de riesgo para la enteritis necrótica, los mecanismos de virulencia del *Clostridium perfringens* permanecen inciertos. El objetivo de este estudio fue la utilización de un modelo experimental de enteritis necrótica producido por la coinfección de *Eimeria maxima* y *Clostridium perfringens* para investigar los parámetros patológicos e inmunológicos de la enfermedad. Pollos de engorde infectados con *Eimeria maxima* y *Clostridium perfringens* mostraron un mayor número de lesiones intestinales severas que las aves que recibieron sólo *Eimeria maxima* o sólo *Clostridium perfringens.* Adicionalmente, la coinfección con *Eimeria maxima* y *Clostridium perfringens* incrementó el número de bacterias (*Clostridium perfringens*) presentes en el intestino, en comparación con las aves desafiadas con solo *Clostridium perfringens*. La coinfección con *Eimeria maxima* y *Clostridium perfringens* disminuyó la expresión del gen de la óxido nítrico sintetasa inducida por la infección con solo *Eimeria maxima*, lo que conllevó a niveles mas bajos de oxido nítrico en plasma. La expresión en el intestino de una serie de genes de citoquinas y quimoquinas posteriores a la coinfección con *Eimeria maxima* y *Clostridium perfringens* mostró una respuesta mixta dependiendo del producto analizado y del tiempo transcurrido desde la infección. En general, se suprimieron los siguientes factores: INF-α, INF-γ, INF-β, IL2, IL12, IL13, IL17 y el TGF-β4, mientras que las IL8, IL10, IL15 y el LITAF se incrementaron durante la coinfección con *Eimeria maxima* y *Clostridium perfringens* en comparación con las aves desafiadas sólo con la *Eimeria maxima* o sólo con el *Clostridium perfringens.* Estos resultados se discuten en el contexto de que *Eimeria maxima* y *Clostridium perfringens* actúan sinergísticamente para crear un fenotipo de la enfermedad que genera una respuesta de citoquinas y quimoquinas diferente a la producida por la infección con el patógeno individual.

Key words: necrotic enteritis, *Clostridium perfringens*, *Eimeria maxima*, innate immunity, coccidiosis

Abbreviations: CP = *Clostridium perfringens*; EM = *Eimeria maxima*; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IELs = intestinal intraepithelial lymphocytes; IL = interleukin; iNOS = inducible nitric oxide synthase; LITAF = lipopolysaccharide-induced TNF-α factor; TGF-β4 = transforming growth factor-β4; TNFSF15 = tumor necrosis factor superfamily 15

Clostridium perfringens (CP) is the causative agent of necrotic enteritis (NE), a disease of increasing economic importance to the global poultry industry (31,33). One of the primary factors considered to be responsible for the rising incidence of NE is reduction or discontinuation of the use of antibiotics and growth-promoter feed additives in commercial poultry production. Although CP occurs naturally in the gut in healthy chickens, its potential to produce overt NE is dependent upon a number of factors, especially the presence of other enteric pathogens (33). In particular, coinfection with certain species of *Eimeria*, such as *E. maxima* (EM), has been strongly implicated in promoting NE (34). However, the molecular and cellular mechanisms by which *Eimeria* potentiates CP-induced NE remain to be elucidated, and there is an urgent need to understand better the host–pathogen interactions if this disease is to be effectively managed.

[B]Corresponding author. E-mail: hlilleho@anri.barc.usda.gov.

Table 1. Experimental scheme.

Group	Treatment	EM (day)	CP (day)	Body weight (days)	Lesion score (days)	CP counting (days)	Plasma NO (days)	IEL RNA collection (days)	Percent survival[A] (live/dead)
1	No infection	–	–	21,26,28	21,27,28,31	26,27,28	21,26,27,28	27,28,31	100% (25/25)
2	EM infection[B]	21	–	21,26,28	21,27,28,31	26,27,28	21,26,27,28	27,28,31	100% (25/25)
3	CP infection[C]	–	26	21,26,28	21,27,28,31	26,27,28	21,26,27,28	27,28,31	96.0% (24/25)
4	EM/CP coinfection[BC]	21	26	21,26,28	21,27,28,31	26,27,28	21,26,27,28	27,28,31	88.0% (22/25)

[A]Percent survival was determined at day 31 (day 5 following CP infection).
[B]Chickens were orally infected with 5.0×10^4 sporulated oocysts/bird of EM at day 21 of age as described (16).
[C]Chickens were orally infected with 1.0×10^9 CFU/bird of a mixture of 5 strains of CP producing toxin A at day 26 of age (day 5 following EM infection) as described (35).

Several different *Eimeria*/CP experimental coinfection procedures have been described in order to develop an animal model of NE (reviewed in reference 31). In the present study, we developed a NE experimental model involving oral exposure of broilers to sporulated oocysts of EM followed 5 days later by gavage challenge with live CP bacteria. One or two days postinfection with CP, intestinal pathology, growth performance, and innate immune responses at the molecular level were compared between chickens coinfected with EM plus CP, animals given a single challenge of EM or CP, and noninfected controls. Our results demonstrated that EM/CP coinfection resulted in more severe intestinal pathology, reduced body weight gain, increased numbers of intestinal CP bacteria, and altered cytokine/chemokine expression compared with chickens exposed to EM or CP alone.

MATERIALS AND METHODS

Animals, CP, and EM. Straight-run, day-old commercial broiler chickens from a local hatchery received *ad libitum* autoclaved drinking water and irradiated feed. All birds were fed a nonmedicated commercial basal ration (Southern States™) of 17% crude protein from 1 to 26 days of age. At 26 days of age, the feed was replaced with a commercial nonmedicated feed containing 24% crude protein (Dumor™) starter feeds. A CP inoculum was prepared using Robertson's cooked meat broth incubated at 37 C, in anaerobic jars with the use of the Mitsubishi AnaeroPak® system. The CP titer is approximately 7–8 log colony forming units (CFU)/ml. EM oocysts from a commercial broiler source were isolated as described (16).

Experimental infections. Three-week-old chickens were divided into four groups (25/group, Table 1). Group 1 consisted of noninfected controls. Animals in groups 2 and 4 were orally infected with 5.0×10^4 sporulated oocysts of EM at day 21 as described (16). Five days later, chickens in groups 3 and 4 were orally infected with 1.0×10^9 CFU of CP. At five time points, five birds from each group were sacrificed for measurement of body weights (days 21, 26, and 28), intestinal lesion scoring (days 21, 27, 28, and 31), intestinal CP counting (days 26, 27, and 28), measurement of plasma NO levels (days 21, 26, 27, and 28), and collection of intestinal intraepithelial lymphocytes (IELs) (days 27, 28, and 31). All protocols were approved by the Institutional Animal Care and Use Committees of the Beltsville Agricultural Research Institute and the University of Delaware.

Bacterial counts in intestinal tissues. Intestinal CP bacteria were enumerated based on the Brown and Hopps's Gram staining protocol (4). Briefly, segments of intestinal jejunum were fixed in 10% buffered neutral formalin and embedded in paraffin, and 5-μm sections were stained (Electron Microscopy Sciences, Hatfield, PA). Gram-positive bacteria were stained dark blue by light microscopy. Bacteria in five fields of view of each tissue section (five birds per group) were counted with the use of a 40× objective.

Analysis of plasma nitrate and nitrite. Chickens were bled by cardiac puncture with the use of Monovette tubes (Sarstedt, Newton, NC) containing 1.6 mg/ml of potassium EDTA. Blood samples were centrifuged; plasmas were collected and spin filtered through 30-kDa molecular weight cutoff filters (Centricon-30, Millipore, Billerica, MA) for 2 hr at 20 C. The clear colorless filtrates were diluted 1:3 (v:v) with water and analyzed colorimetrically in a 96-well format for total NO_2^- with the use of the Griess reaction after reducing NO_3^- to NO_2^- with nitrate reductase (1,32).

Table 2. Sequence of primer sets used for quantitative real time RT-PCR.

RNA target	Primer sequences		Product size (bp)	Accession number
	Forward	Reverse		
GAPDH	5′-GGTGGTGCTAAGCGTGTTAT	5′-ACCTCTGTCATCTCTCCACA	264	K01458
IFN-α	5′-GACATCCTTCAGCATCTCTTCA	5′-AGGCGCTGTAATCGTTGTCT	238	AB021154
IFN-γ	5′-AGCTGACGGTGGACCTATTATT	5′-GGCTTTGCGCTGGATTC	259	Y07922
IL-1β	5′-TGGGCATCAAGGGCTACA	5′-TCGGGTTGGTTGGTGATG	244	Y15006
IL-2	5′-TCTGGGACCACTGTATGCTCT	5′-ACACCAGTGGGAAACAGTATCA	256	AF000631
IL-6	5′-CAAGGTGACGGAGGAGGAC	5′-TGGCGAGGAGGGATTTCT	254	AJ309540
IL-8	5′-GGCTTGCTAGGGGAAATGA	5′-AGCTGACTCTGACTAGGAAACTGT	200	AJ009800
IL-10	5′-CGGGAGCTGAGGGTGAA	5′-GTGAAGAAGCGGTGACAGC	272	AJ621614
IL-12	5′-AGACTCCAATGGGCAAATGA	5′-CTCTTCGGCAAATGGACAGT	274	NM_213571
IL-13	5′-CCAGGGCATCCAGAAGC	5′-CAGTGCCGGCAAGAAGTT	256	AJ621735
IL-15	5′-TCTGTTCTTCTGTTCTGAGTGATG	5′-AGTGATTTGCTTCTGTCTTTGGTA	243	AF139097
IL-16	5′-TCCCTCTGCAAAATGGTCA	5′-TCGCGATCTCAGGTTGTGT	271	AJ508678
IL-17	5′-CTCCGATCCCTTATTCTCCTC	5′-AAGCGGTTGTGGTCCTCAT	292	AJ493595
LITAF	5′-TGTGTATGTGCAGCAACCCGTAGT	5′-GGCATTGCAATTTGGACAGAAGT	229	AY765397
K60	5′-ATTTCCTCCTGCCTCCTACA	5′-GTGACTGGCAAAAATGACTCC	228	AF277660
TGF-β4	5′-CGGGACGGATGAGAAGAAC	5′-CGGCCCACGTAGTAAATGAT	258	M31160
TNFSF15	5′-CCTGAGTATTCCAGCAACGCA	5′-ATCCACCAGCTTGATGTCACTAAC	292	NM_01024578
iNOS	5′-TGGGTGGAAGCCGAAATA	5′-GTACCAGCCGTTGAAAGGAC	241	U46504

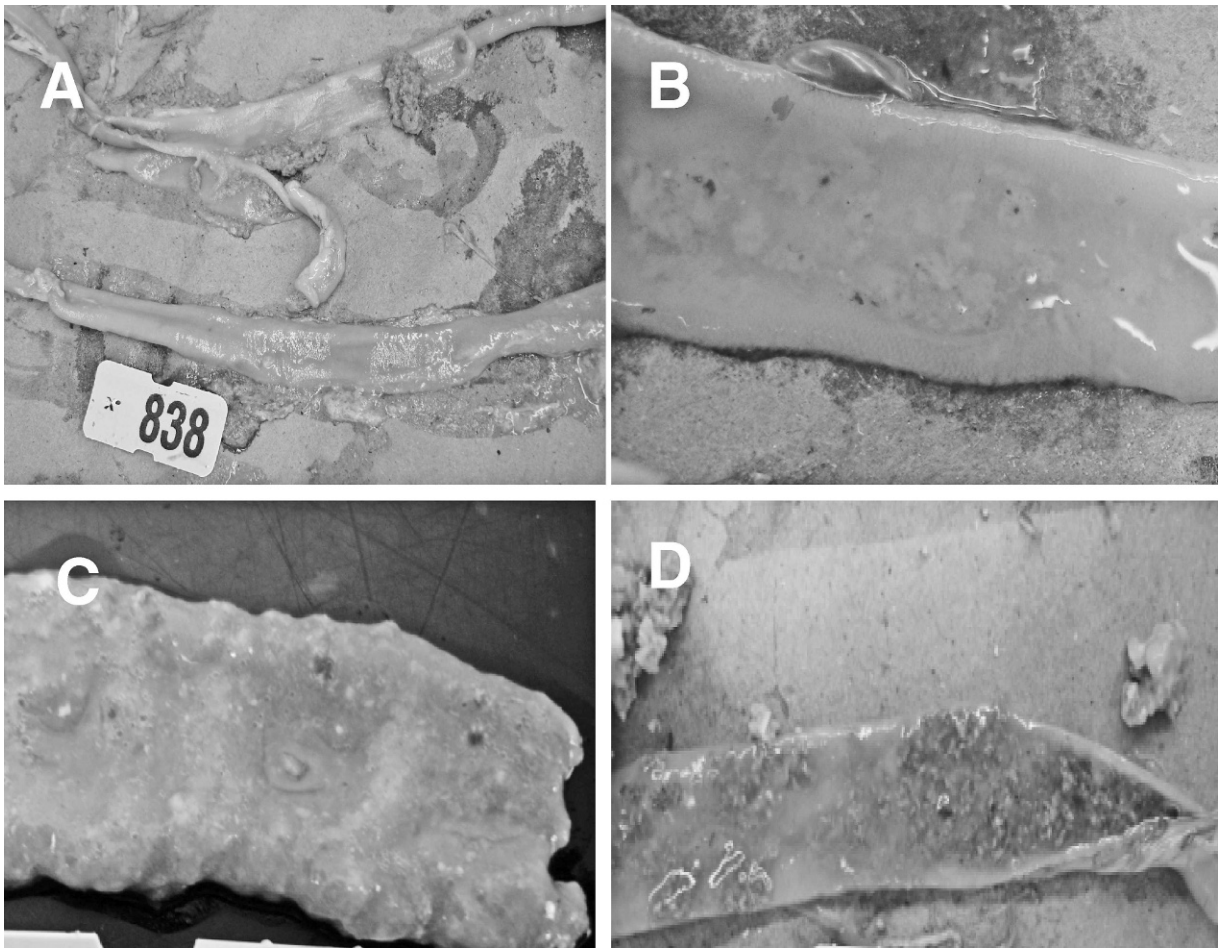

Fig. 1. Gross pathology of intestines from chickens infected with EM and/or CP. (A) Jejunum of noninfected control bird. (B) Jejunum of chicken at day 7 following infection with EM alone. (C) Jejunum of chicken coinfected with EM and CP at day 1 following CP challenge. (D) Jejunum of chicken infected with CP alone.

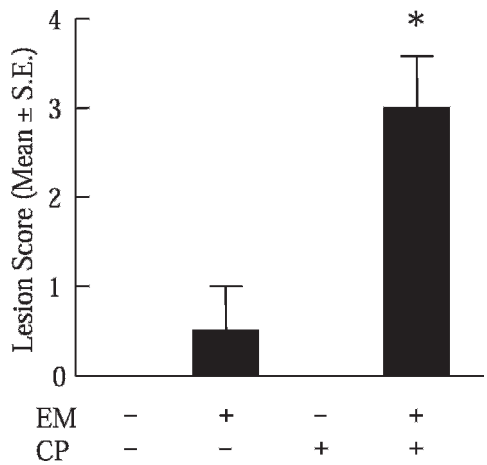

Fig. 2. Intestinal lesion scores of chickens infected with EM and/or CP. Chickens were noninfected, infected with EM or CP alone, or coinfected with EM and CP, and jejunums were examined at day 2 following CP infection. Lesions were scored on a scale of 0 (no lesions) to 4 (most severe lesions) as described (24). Each bar represents the mean ± SE of five birds. The asterisk denotes significantly increased lesion score in EM/CP coinfected chickens compared with chickens infected with EM or CP alone ($P < 0.05$). (*$P < 0.05$).

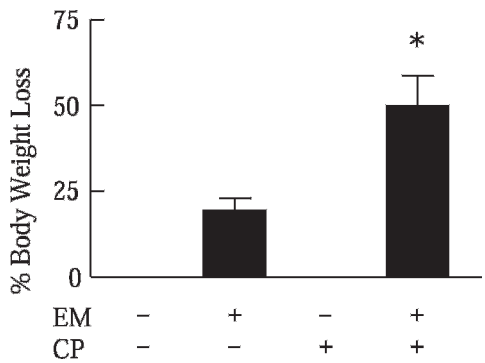

Fig. 3. Body weight loss of chickens infected with EM and/or CP. Chickens were noninfected, infected with EM or CP alone, or coinfected with EM and CP and body weights were measured at the time of EM challenge and at day 2 postchallenge with CP (7-day interval). The percent of body weight loss (body weight differences between day 21 and day 28 of age) of each bird compared with the noninfected control birds was determined. Each bar represents the mean ± SE of five birds. The asterisk denotes significantly increased percent of chickens exhibiting reduction in weight gain following EM/CP coinfection compared with chickens infected with EM or CP alone ($P < 0.05$).

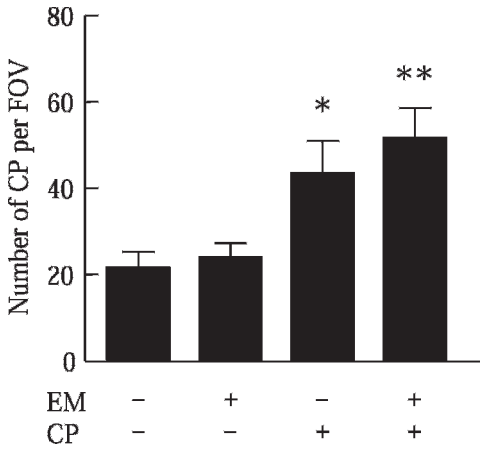

Fig. 4. Intestinal CP in chickens infected with EM and/or CP. Chickens were noninfected, infected with EM or CP alone, or coinfected with EM and CP and the number of CP bacteria in the jejunum at day 2 following CP challenge were measured as described (4). Each bar represents the mean ± SE of 25 fields of view (FOV). The asterisks denote significantly increased numbers of CP following EM/CP coinfection (**$P < 0.01$) or following CP alone (*$P < 0.05$) compared with noninfected chickens or infected with EM alone.

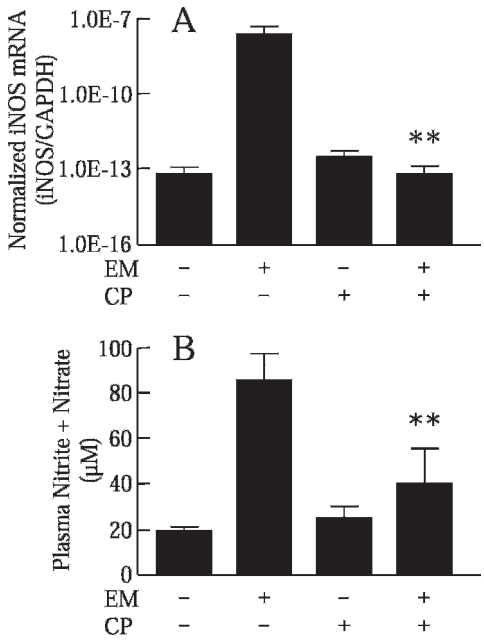

Fig. 5. Levels of iNOS mRNA and plasma NO in chickens infected with EM and/or CP. Chickens were noninfected, infected with EM or CP alone, or coinfected with EM and CP. (A) iNOS transcripts in intestinal IELs were measured at day 1 after CP infection by quantitative real time RT-PCR and normalized to GAPDH mRNA levels. (B) Plasma nitrite + nitrate levels were measured at day 2 after CP infection. Each bar represents the mean ± SE of five birds. The asterisks denote significantly decreased levels of iNOS transcripts or NO following EM/CP coinfection compared with chickens infected with EM alone ($P < 0.01$).

Total RNA extraction and cDNA synthesis. Intestinal tissues were collected between the Meckel's diverticulum to the ileac region, cut longitudinally, and washed three times with ice-cold Hank's balanced salt solution containing 100 U/ml of penicillin and 100 μg/ml of streptomycin (Sigma, St. Louis, MO). Tissues were immediately submerged in RNAlater solution (Ambion, Austin, TX) and the mucosal layer was scraped away with a scalpel. Total RNA from IELs was isolated with the use of TRIzol (Invitrogen, Carlsbad, CA) as described (16). Five micrograms of total RNA from each sample were reverse transcribed into cDNA with the use of the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR primers for iNOS, cytokines, chemokines, and GAPDH are listed in Table 2. Transcript levels of the indicated genes were normalized to those of GAPDH, a less abundant mRNA compared with 28S RNA. In preliminary experiments with three selected transcripts, no differences in expression patterns of IFN-γ, MyD88, or MIP-1β were noted when normalized to GAPDH mRNA or 28S RNA (data not shown). Amplification and detection of transcripts were carried out with the use of equivalent amounts of cDNA with the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene) as described (16,23). Standard curves were generated with the use of $\log_{10}$ dilutions of standard RNA, and the levels of individual transcripts were normalized to those of GAPDH by Q-gene software (Stratagene) (26). Each analysis was performed in triplicate. To normalize mRNA expression levels between samples within individual experiments, the mean threshold cycle value (C_t) for the corresponding target and GAPDH products were calculated by pooling values from all samples in that experiment.

Statistical analysis. Mean ± SE values for each group were calculated, differences between groups were analyzed by the Dunnet multiple-comparison test or the Tukey–Kramer multiple comparison test with the use of Graphpad Prism 4 software (Graphpad, San Diego, CA), and considered significant at $P < 0.05$.

RESULTS

Coinfection of broilers with EM and CP augments intestinal pathology. In this NE experimental system that we developed using coinfection of EM and CP, 88% (22/25) of chickens survived the EM/CP coinfection protocol (Table 1). Three birds in EM/CP group died 24 hr postinfection due to acute enterotoxemia and showed severe classic NE signs and focal necrotic enteritis lesions. As shown in Fig. 1, intestinal gross pathology in chickens coinfected with EM plus CP was substantially more severe compared with birds infected with EM alone. Gross and microscopic examination of intestinal lesions of birds infected with EM alone showed parasites, and lesions typical of coccidial infection with bumpy intestinal mucosal thickening in the jejunum that were observed beginning at 7 days postinfection (Fig. 1B). By contrast, infection by CP produced minimal intestinal pathology, with only approximately 10% of chickens examined displaying evidence of necrotic lesions (Fig. 1D). Following EM/CP coinfection, however, both the severity and time of appearance of lesions were dramatically increased compared with singly infected chickens. Thus, at day 1 following CP infection in the coinfected animals (day 6 after EM infection), coccidial lesions were readily observed in the jejunum (Fig. 1C). By day 2 after CP infection, all animals demonstrated moderate to severe lesions. One coinfection postmortem sample in particular exhibited significant necrotic lesions characterized by a intestinal ballooning, a watery mucoid gut lining, and fibrinonecrotic membrane formation at day 2 following CP infection (unpublished data). A more quantitative analysis of lesion scores measured in the four experimental groups confirmed the results of the gross pathology observations (Fig. 2).

Coinfection with EM and CP reduces growth performance. The major clinical manifestation of intestinal damage due to *Eimeria* infection is reduction of body weight gain due to malabsorption of nutrients. Therefore, body weights were determined in noninfected control, singly infected, and coinfected animals. Body weight gains of noninfected control birds or birds challenged with CP alone were

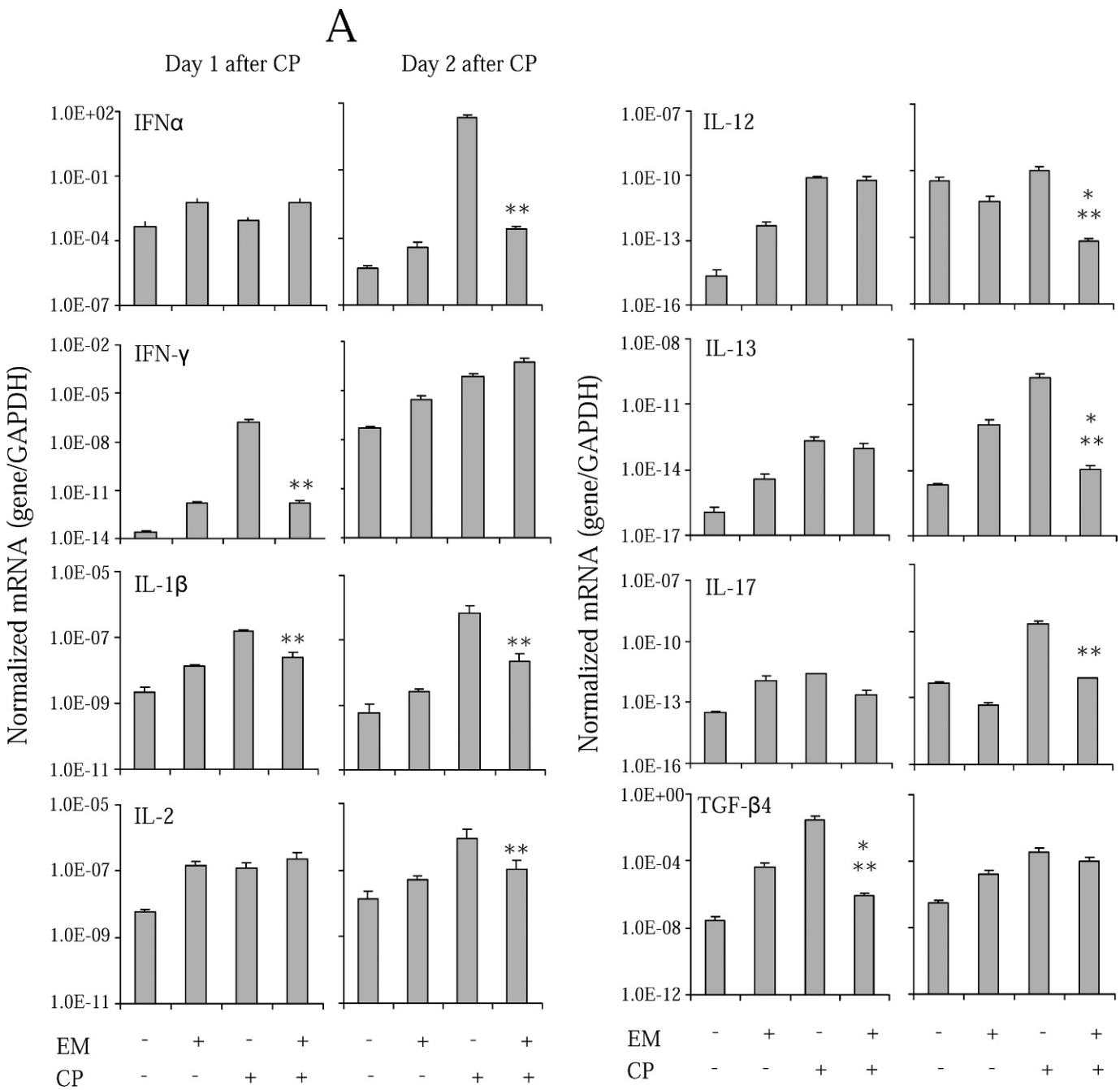

Fig. 6. Levels of transcripts coding for cytokine, chemokine, and other immune-related molecules in chickens infected with EM and/or CP. Chickens were noninfected, infected with EM or CP alone, or coinfected with EM and CP, transcripts for the indicated molecules were measured at 1 or 2 days after CP infection, and normalized to GAPDH transcripts. (A) Transcript levels that were decreased in EM/CP coinfected chickens compared with chickens infected with EM or CP alone. The asterisks denote significantly decreased levels of the indicated transcripts following EM/CP coinfection compared with chickens infected with EM (*) or CP (**) alone ($P < 0.05$). (B) Transcript levels that were increased in EM/CP coinfected chickens compared with chickens infected with EM or CP alone. The asterisks denote significantly increased levels of the indicated transcripts following EM/CP coinfection compared with chickens infected with EM (*) or CP (**) alone ($P < 0.05$). (C) Transcripts levels that were decreased at day 1 and increased at day 2 after CP infection in EM/CP coinfected chickens compared with chickens infected with EM or CP alone. The asterisks denote significantly changed levels of the indicated transcripts following EM/CP coinfection compared with chickens infected with EM (*) or CP (**) alone ($P < 0.05$). (D) Transcript levels that were not changed in EM/CP coinfected chickens compared with chickens infected with EM or CP alone. Each bar represents the mean ± SE of triplicate samples in all experiments.

approximately doubled between 21 and 28 days of age in the control and CP-challenged birds. By contrast, 20% of birds exhibited severe loss (>50%) of body weight gain following infection by EM alone. Moreover, when chickens were coinfected with EM and CP, 50% of the animals exhibited severe loss of body weight gain (Fig. 3). The percentage of animals exhibiting >50% loss of weight gain in the coinfected chickens compared with those challenged with EM alone was significantly increased ($P < 0.05$). Combined with the results in Figs. 1 and 2, these results suggest that EM/CP coinfection plays an important role as a predisposing factor in NE by damaging the chicken intestinal mucosa and leading to decreased growth performance.

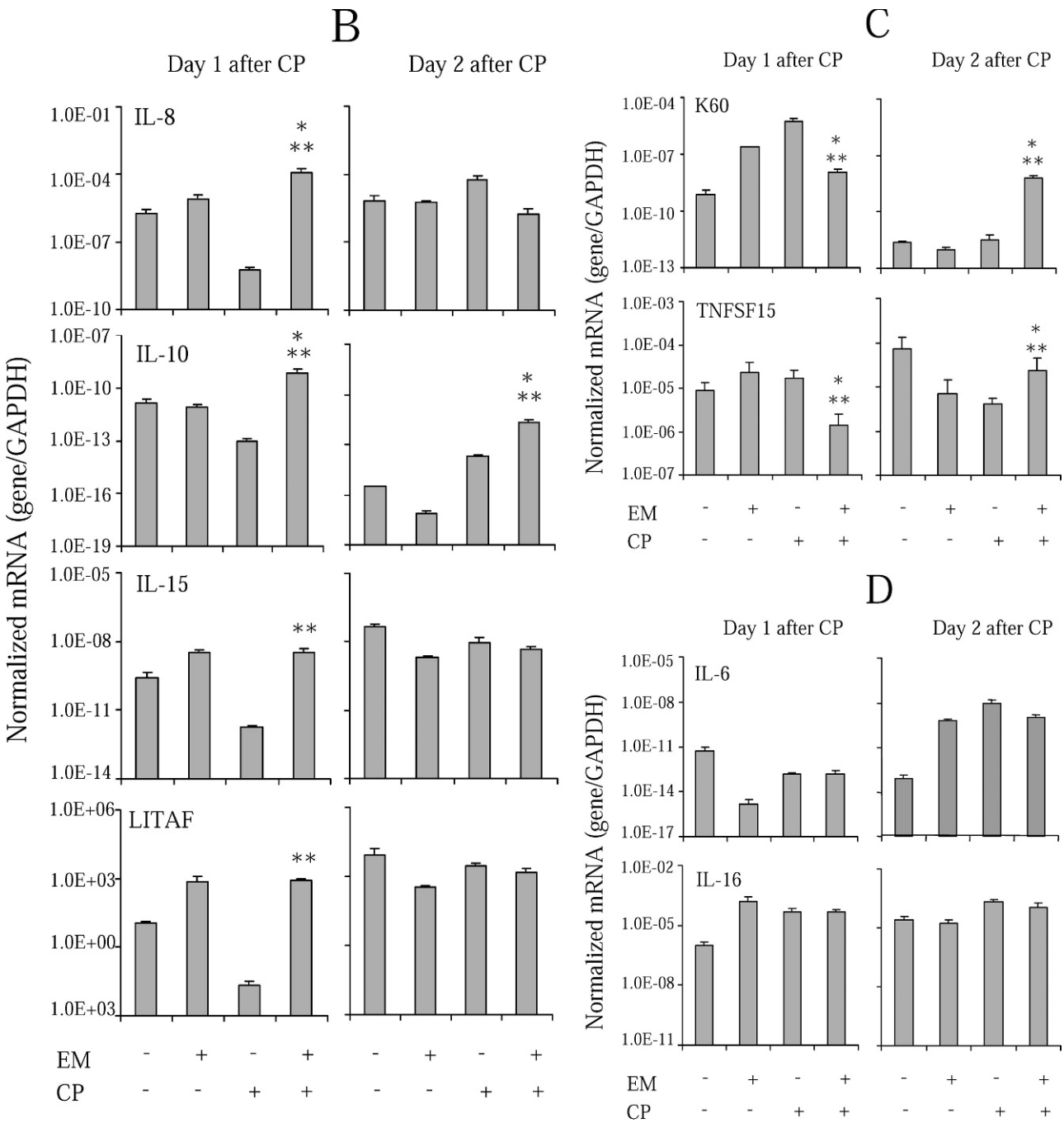

Fig. 6. Continued.

Coinfection with EM and CP increases the number of intestinal CP. Next, we determined the effect of EM/CP coinfection on the number of CP bacteria in the gut. Compared with chickens infected with CP alone, the number of bacteria seen in the jejunum of coinfected animals was increased approximately 20%, and this increase was statistically significant ($P < 0.05$) (Fig. 4). Interestingly, however, EM infection by itself did not alter the number of nonpathogenic CP strains naturally resident in the gut, suggesting that the effect of coccidia infection was specific to the pathogenic bacteria given during experimental inoculation.

Coinfection with EM and CP reduces intestinal iNOS expression and NO levels. Our previous studies demonstrated that infection of chickens with *Eimeria* spp. induced iNOS expression *in vitro* (9,10,21) and increased plasma NO levels *in vivo* (2). Therefore, it was of interest to determine the effects of EM/CP coinfection on intestinal iNOS expression and plasma NO levels. As shown in Fig. 5A, the levels of iNOS transcripts were significantly increased in intestinal IELs of birds infected with EM alone, whereas CP alone had no effect on intestinal iNOS gene expression. In dually infected birds, the levels of iNOS transcripts were equivalent to those seen in noninfected or CP-only infected animals. Thus, the effect of CP was to repress the expression of iNOS induced by EM. As shown in Fig. 5B, the levels of plasma NO showed a similar trend, that is, increased levels in EM only infected animals and significantly reduced levels following EM/CP confection.

Coinfection with EM and CP alters intestinal expression of immune-related genes. Infection of chickens with coccidia parasites results in the production of a diverse array of immune-response–

Table 3. Summary of cytokine/chemokine responses to EM/CP coinfection.

Type of response[A]	Type of cytokine/chemokine	Cytokine or chemokine	Decreased or increased	Fold change[B]
Uniform response	Proinflammatory cytokine	IFN-α	Decreased	1.1×10^5
		IL-1β	Decreased	3.3×10^2
		IL-17	Decreased	3.6×10^3
		LITAF	Increased	4.1×10^4
	Th1 cytokine	IFN-γ	Decreased	9.2×10^6
		IL-2	Decreased	2.0×10^0
		IL-12	Decreased	6.8×10^4
		TGF-β4	Decreased	3.3×10^5
		IL-10	Increased	6.9×10^3
		IL-15	Increased	1.7×10^3
	Th2 cytokine	IL-13	Decreased	5.7×10^5
	Chemokine	IL-8	Increased	2.3×10^4
Mixed response	Proinflammatory cytokine	TNFSF15	Decreased	7.8×10^2
			Increased	5.6×10^0
	Chemokine	K60	Decreased	2.0×10^3
			Increased	1.9×10^3
No change	Proinflammatory cytokine	IL-6	NA[C]	NA
	Th1 cytokine	IL-16	NA	NA

[A]Uniform response indicates either increased or decreased, and mixed response denotes increased and decreased or decreased and increased response at days 1 and 2 post CP infection.
[B]Compared to single infection groups.
[C]NA, not applicable.

related molecules in the gut, including those analyzed in the current study (5,6,7,8,14,15,16,19,20,21,22,24,29). Therefore, in light of the finding that CP suppressed EM-induced iNOS expression, we sought to determine whether or not EM/CP coinfection altered the expression of other immune-related genes compared with infection by EM or CP alone. For this analysis, we used a panel of 16 genes encoding molecules associated with a proinflammatory (IFN-α, IL-1β, IL-6, IL-17, LITAF, TNFSF15) or anti-inflammatory (TGF-β4) response, Th1 cytokine response (IFN-γ, IL-2, IL-10, IL-12, IL-15, IL-16), a Th2 cytokine (IL-13), and chemokines (IL-8, K60). As shown in Fig. 6A, the levels of eight transcripts (IFN-α, IFN-γ, IL-1β, IL-2, IL-12, IL-13, IL-17, TGF-β4) were significantly repressed following dual EM/CP infection at either 1 or 2 days following CP exposure, compared with single EM or CP infections. By contrast, 4 mRNAs (IL-8, IL-10, IL-15, LITAF) were significantly upregulated under the same experimental conditions (Fig. 6B). Two transcripts (K60, TNFSF15) were upregulated at day 1 but downregulated at day 2 following CP exposure in the coinfected animals (Fig. 6C), whereas two others (IL-6, IL-16) showed no altered response to coinfection *vs*. single infections (Fig. 6D). Table 3 summarizes these results according to the type of cytokine or chemokine gene examined and lists the maximum fold change of gene expression that was measured in the coinfected chickens *vs*. single infections. In conclusion, it is apparent that a complex and dynamic response of immune-related gene expression occurs in the gut during experimental NE.

DISCUSSION

NE is an emerging and devastating poultry disease. Compared with the well-established experimental methods to cause coccidiosis, however, lack of a reproducible NE disease model has been a major obstacle in understanding the basic parameters of immunity in this disease. Recently several NE experimental models utilizing oral or cloacal CP challenge routes have been reported (18,27). Other disease models have utilized coadministration of *Eimeria* spp. and CP under the assumption that the host response to infection by both pathogens may be qualitatively and/or quantitatively different from that due to CP alone (3,17,34). We experimentally reproduced NE with the use of oral challenges that constitute more natural routes of infection by EM and CP, and document for the first time some of the pathologic and immunologic parameters associated with this coinfection model. These results can be summarized as follows. First, intestinal damage and loss of body weight gain during EM/CP coinfection was more severe than that produced by EM alone, indicating that CP augments the tissue destructive properties of coccidia parasites. Second, the number of CP bacteria in the gut during EM/CP coinfection was greater than that observed following challenge with the same number of bacteria alone, suggesting that destruction of the gut integrity by EM facilitated the propagation of CP. Finally, expression of a large repertoire of genes involved in mediating innate immunity was altered during coadministration of both pathogens compared with either alone, suggesting that the host inflammatory response is fundamentally different during dual infection.

Coccidiosis, a major intestinal parasitic disease of poultry, induces a cell-mediated immune response against the etiologic agent of the disease, *Eimeria* (20,22). The expression levels of gene transcripts encoding proinflammatory, Th1, and Th2 cytokines, as well as chemokines, were measured in IELs after *E. maxima*, *E. tenella*, *and E. acervulina* infections (14,16). Compared with uninfected controls, transcripts of many proinflammatory cytokines were increased following primary infection. By contrast, following secondary infection, proinflammatory mRNAs levels were relatively unchanged. We conclude that coccidiosis induces a diverse and robust primary cytokine/chemokine response, but a more subdued secondary response.

Despite multiple studies that have investigated cellular and humoral immune responses to CP infection in chickens, particularly from the perspective of potential NE vaccines, relatively few reports have appeared describing cytokine and chemokine responses in these animals (11). The results illustrated in Fig. 6 demonstrate that CP infection by itself induces the expression of several important immune mediators in the gut, namely, IFN-α, IFN-γ, IL-1β, IL-2, IL-12, IL-13, IL-17 and TGF-β4. However, expression levels of all of these cytokines were significantly reduced following EM/CP coinfection. In some cases, the level of cytokine expression after

coinfection was less than that seen in either of the singly infected animals (e.g., IL-12 and IL-13 at 2 days following CP challenge). In other cases, reduced expression of a particular transcript may be dependent upon inhibition of an upstream mediator. For example, decreased iNOS in coinfected animals may be secondary to reduced IFN-γ, because the latter induces iNOS in immune cells (19). IL-2 is a Th1-type cytokine that plays a central role in adaptive immunity and IL-12 is an important regulatory cytokine required for the initiation and regulation of cellular immunity (25). IL-12 regulates the differentiation of naive T cells into Th1-type cells, which is crucial for resistance to many microbial pathogens (12). On the other hand, TGF-β4, which is generally considered as an anti-inflammatory cytokine (19), also was repressed during EM/CP coinfection. The function of TGF-β4, however, is dependent on its concentration at local sites of infection, being proinflammatory at low concentrations and anti-inflammatory at high concentrations (6,19,28). For example, avian TGF-β4 has opposite effects on IFN-γ production by natural killer cells in the gut depending on its concentration. Current studies in our laboratory are directed at determining the pro- or anti-inflammatory role of TGF-β4 during EM/CP coinfection. In summary, therefore, it is apparent that pre-exposure to EM represses the ability of CP to induce an effective inflammatory cytokine response, which may account for the exacerbated pathologic findings and increased CP colonization seen during experimental NE.

Another group of transcripts coding for at least four immune effecter molecules were upregulated during EM/CP coinfection compared with EM or CP alone. IL-8 is a CXC chemokine that attracts leukocytes to sites of inflammation on mucosal surfaces. IL-10 is a pleiotropic Th2-type cytokine involved in innate and adaptive immune responses and functions to inhibit the production of IL-12 by activated macrophages, $CD4^+$ and $CD8^+$ T lymphocytes (13,30). Increased IL-10 following dual infection might actively regulate Th2 cell bias during experimental NE. IL-15 is structurally homologous to IL-2, and we speculate that IL-15 upregulation may compensate for IL-2 downregulation during EM/CP dual infections. Finally, LITAF is a transcription factor that upregulates TNF-α gene expression and was shown to be identical to the p53-inducible gene 7 that was upregulated during p53-mediated apoptosis (35). Our previous study demonstrated that chicken LITAF mRNA was predominantly expressed in spleen and intestinal IELs, and expression was upregulated following *in vitro* stimulation of macrophages with *Escherichia coli* or *Salmonella* Typhimurium endotoxin, or with *E. acervulina*, *E. maxima*, or *E. tenella* oocysts (15).

Taken together, the data presented in this report establish that a synergistic relationship exists between EM and CP during the course of experimental NE resulting in an intestinal disease phenotype that is qualitatively and quantitatively different from that produced by infection by either enteric microorganism alone. These results provide the foundation for future analysis of innate and adaptive immune responses during NE with the use of novel, state-of-the-art tools recently developed for avian species, including macrophage and intestinal IEL microarrays, quantitative RT-PCR, flow cytometry, and immunohistochemical protocols. Moreover, this comparative coinfection model could be applied with the use of additional coccidia species along CP or adapted to different combinations of parasitic, bacterial, and viral pathogens in other disease settings.

REFERENCES

1. Allen, P. C. Nitric oxide production during *Eimeria tenella* infections in chickens. Poult. Sci. 76:810–813. 1997.

2. Allen, P. C., and H. S. Lillehoj. Genetic influence on nitric oxide production during *Eimeria tenella* infections in chickens. Avian Dis. 42:397–403. 1998.

3. Baba, E., A. L. Fuller, J. M. Gilbert, S. G. Thayer, and L. R. McDougald. Effects of *Eimeria brunetti* infection and dietary zinc on experimental induction of necrotic enteritis in broiler chickens. Avian Dis. 36:59–62. 1992.

4. Brown, R. C., and H. C. Hopps. Staining of bacteria in tissue sections: a reliable Gram stain method. Am. J. Clin. Pathol. 60:234–240. 1973.

5. Choi, K. D., and H. S. Lillehoj. Role of chicken IL-2 on γδ T-cells and *Eimeria acervulina*-induced changes in intestinal IL-2 mRNA expression and γδ T-cells. Vet. Immunol. Immunopathol. 73:309–321. 2000.

6. Choi, K. D., H. S. Lillehoj, and D. S. Zalenga. Changes in local IFN-γ and TGF-β4 mRNA expression and intraepithelial lymphocytes following *Eimeria acervulina* infection. Vet. Immunol. Immunopathol. 71:263–275. 1999.

7. Dalloul, R. A., T. W. Bliss, Y. H. Hong, I. Ben-Chouikha, D. W. Park, C. L. Keeler, and H. S. Lillehoj. Unique responses of the avian macrophage to different species of *Eimeria*. Mol. Immunol. 44:558–566. 2007.

8. Dalloul, R. A., and H. S. Lillehoj. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Rev. Vaccines 5:143–163. 2006.

9. Dalloul, R. A., H. S. Lillehoj, D. M. Klinman, X. Ding, W. Min, R. A. Heckert, and E. P. Lillehoj. In ovo administration of CpG oligodeoxynucleotides and the recombinant microneme protein MIC2 protects against *Eimeria* infections. Vaccine 23:3108–3113. 2005.

10. Dalloul, R. A., H. S. Lillehoj, J. S. Lee, S. H. Lee, and K. S. Chung. Immunopotentiating effect of a *Fomitella fraxinea*–derived lectin on chicken immunity and resistance to coccidiosis. Poult. Sci. 85:446–451. 2006.

11. Degen, W. G., H. I. van Zuilekom, N. C. Scholtes, N. van Daal, and V. E. Schijns. Potentiation of humoral immune responses to vaccine antigens by recombinant chicken IL-18 (rChIL-18). Vaccine 23:4212–4218. 2005.

12. Eldaghayes, I., L. Rothwell, A. Williams, D. Withers, S. Balu, F. Davison, and P. Kaiser. Infectious bursal disease virus: strains that differ in virulence differentially modulate the innate immune response to infection in the chicken bursa. Viral Immunol. 19:83–91. 2006.

13. Groux, H., and F. Powrie. Regulatory T cells and inflammatory bowel disease. Immunol. Today 20:442–445. 1999.

14. Hong, Y. H., H. S. Lillehoj, S. H. Lee, R. A. Dalloul, and E. P. Lillehoj. Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections. Vet. Immunol. Immunopathol. 114:209–223. 2006.

15. Hong, Y. H., H. S. Lillehoj, S. H. Lee, D. W. Park, and E. P. Lillehoj. Molecular cloning and characterization of chicken lipopolysaccharide-induced TNF-α factor (LITAF). Dev. Comp. Immunol. 30:919–929. 2006.

16. Hong, Y. H., H. S. Lillehoj, E. P. Lillehoj, and S. H. Lee. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria maxima* infection of chickens. Vet. Immunol. Immunopathol. 114:259–272. 2006.

17. Jackson, M. E., D. M. Anderson, H. Y. Hsiao, G. F. Mathis, and D. W. Fodge. Beneficial effect of beta-mannanase feed enzyme on performance of chicks challenged with *Eimeria* sp. and Clostridium perfringens. Avian Dis. 47:759–763. 2003.

18. Kaldhusdal, M., M. Hofshagen, A. Lovland, H. Langstrand, and K. Redhead. Necrotic enteritis challenge models with broiler chickens raised on litter: evaluation of preconditions, *Clostridium perfringens* strains and outcome variables. FEMS Immunol. Med. Microbiol. 24:337–343. 1999.

19. Laurent, F., R. Mancassola, S. Lacroix, R. Menezes, and M. Naciri. Analysis of chicken mucosal immune response to *Eimeria tenella* and *Eimeria maxima* infection by quantitative reverse transcription-PCR. Infect. Immun. 69:2527–2534. 2001.

20. Lillehoj, H. S. Role of T lymphocytes and cytokines in coccidiosis. Int. J. Parasitol. 28:1071–1081. 1998.

21. Lillehoj, H. S., and G. Li. Nitric oxide production by macrophages stimulated with coccidia sporozoites, lipopolysaccharide, or interferon-γ, and

its dynamic changes in SC and TK strains of chickens infected with *Eimeria tenella*. Avian Dis. 48:244–253. 2004.

22. Lillehoj, H. S., W. Min, and R. A. Dalloul. Recent progress on the cytokine regulation of intestinal immune responses to *Eimeria*. Poult. Sci. 83:611–623. 2004.

23. Min, W., and H. S. Lillehoj. Isolation and characterization of chicken interleukin-17 cDNA. J. Interferon Cytokine Res. 22:1123–1128. 2002.

24. Miyamoto, T., W. Min, and H. S. Lillehoj. Kinetics of interleukin-2 production in chickens infected with *Eimeria tenella*. Comp. Immunol. Microbiol. Infect. Dis. 25:149–158. 2002.

25. Mosmann, T. R., and S. Sad. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today 17:138–146. 1996.

26. Muller, P. Y., H. Janovjak, A. R. Miserez, and Z. Dobbie. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 32:1372–1379. 2002.

27. Olkowski, A. A., C. Wojnarowicz, M. Chirino-Trejo, and M. D. Drew. Responses of broiler chickens orally challenged with *Clostridium perfringens* isolated from field cases of necrotic enteritis. Res. Vet. Sci. 81:99–108. 2006.

28. Omer, F. M., J. A. Kurtzhals, and E. M. Riley. Maintaining the immunological balance in parasitic infections: A role for TGF-β? Parasitol. Today 16:18–23. 2000.

29. Qin, Z. R., A. Arakawa, E. Baba, T. Fukata, T. Miyamoto, K. Sasai, and G. S. Withanage. *Eimeria tenella* infection induces recrudescence of previous *Salmonella enteritidis* infection in chickens. Poult. Sci. 74:1786–1792. 1995.

30. Rothwell, L., J. R. Young, R. Zoorob, C. A. Whittaker, P. Hesketh, A. Archer, A. L. Smith, and P. Kaiser. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J. Immunol. 173:2675–2682. 2004.

31. Van Immerseel, F., J. De Buck, F. Pasmans, G. Huyghebaert, F. Haesebrouck, and R. Ducatelle. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathol. 33:537–549. 2004.

32. Verdon, C. P., B. A. Burton, and R. L. Prior. Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by $NADP^+$ when the Griess reaction is used to assay for nitrite. Anal. Biochem. 224:502–508. 1995.

33. Williams, R. B. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathol. 34:159–180. 2005.

34. Williams, R. B., R. N. Marshall, R. M. La Ragione, and J. Catchpole. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis, coccidiosis and anticoccidial vaccination of chickens. Parasitol. Res. 90:19–26. 2003.

35. Zhu, J., J. Jiang, W. Zhou, K. Zhu, and X. Chen. Differential regulation of cellular target genes by p53 devoid of the PXXP motifs with impaired apoptotic activity. Oncogene 18:2149–155. 1999.

ACKNOWLEDGMENTS

The authors thank Margie Nichols and Eliseo Miramontes for their technical assistance. This project was supported, in part, by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, Grant No. 2004-35204-14798.